DAZAP1, an RNA-binding protein required for development and spermatogenesis, can regulate mRNA translation

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RICHARD W.P. SMITH,[1,2,3] ROSS C. ANDERSON,[1,2,3] JOEL W.S. SMITH,[2,3] MATTHEW BROOK,[1,2] WILLIAM A. RICHARDSON,[1,2] and NICOLA K. GRAY[1,2,4]

[1]MRC Centre for Reproductive Health/MRC Human Reproductive Sciences Unit, Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, Scotland, United Kingdom
[2]MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Western General Hospital, Edinburgh EH4 2XU, Scotland, United Kingdom

ABSTRACT

DAZ-associated protein 1 (DAZAP1) is an RNA-binding protein required for normal growth, development, and fertility in mice. However, its molecular functions have not been elucidated. Here we find that *Xenopus laevis* and human DAZAP1, which are each expressed as short and long forms, act as mRNA-specific activators of translation in a manner that is sensitive to the number of binding sites present within the 3′ UTR. Domain mapping suggests that this conserved function is mainly associated with C-terminal regions of DAZAP1. Interestingly, we find that the expression of xDAZAP1 and its polysome association are developmentally controlled, the latter suggesting that the translational activator function of DAZAP1 is regulated. However, ERK phosphorylation of DAZAP1, which can alter protein interactions with its C terminus, does not play a role in regulating its ability to participate in translational complexes. Since relatively few mRNA-specific activators have been identified, we explored the mechanism by which DAZAP1 activates translation. By utilizing reporter mRNAs with internal ribosome entry sites, we establish that DAZAP1 stimulates translation initiation. Importantly, this activity is not dependent on the recognition of the 5′ cap by initiation factors, showing that it functions downstream from this frequently regulated event, but is modulated by changes in the adenylation status of mRNAs. This suggests a function in the formation of "end-to-end" complexes, which are important for efficient initiation, which we show to be independent of a direct interaction with the bridging protein eIF4G.

Keywords: mRNA-specific translational control; untranslated regions; proline-rich RNA-binding protein (Prrp); gametogenesis; multifunctional RNA-binding proteins

INTRODUCTION

The RNA-binding protein Deleted in Azoospermia (DAZ)–associated protein 1 (DAZAP1), also known as proline-rich RNA-binding protein (Prrp), contains two RNA-recognition motifs (RRMs) and is a member of the family of heterogeneous RNA-binding proteins (Akindahunsi et al. 2005). While most *Dazap1*-deficient (and -hypomorphic) mice die perinatally due to growth defects, the few surviving *Dazap1*-hypomorphic male progeny contain no post-pachytene spermatocytes, indicating roles for Dazap1 in normal growth, development, and spermatogenesis (Hsu et al. 2008). Surviving *Dazap1*-hypomorphic female mice are also sterile (Hsu et al. 2008). However, the molecular functions of Dazap1 and their relative contributions to these phenotypes remain unclear, although its ability to bind RNA (Tsui et al. 2000a; Zhao et al. 2001) suggests that Dazap1 is likely to have a role in regulating post-transcriptional gene expression.

Mammalian DAZAP1 is widely expressed in adults with high levels of expression in testis (Tsui et al. 2000a,b; Dai et al. 2001; Kurihara et al. 2004; Hori et al. 2005; Pan et al. 2005). Within the pachytene spermatocytes and round spermatids of the mouse testis, Dazap1 is predominantly nuclear (Kurihara et al. 2004) and may have nuclear functions as it can play a minor role in mini-gene splicing in human cell lines (Goina et al. 2008; Skoko et al. 2008).

In elongating spermatids in mouse (Kurihara et al. 2004) and during oogenesis in *Xenopus laevis* (Zhao et al. 2001),

[3]These authors contributed equally to this work.
[4]**Corresponding author.**
E-mail Nicola.Gray@ed.ac.uk.
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DAZAP1 is cytoplasmic, suggesting it may also have gene regulatory roles in the cytoplasm. In support of this, DAZAP1 interacts with the actin-associated protein profilin (Zhao et al. 2001) and forms part of a large protein complex bound to the 3′ UTR of mRNAs, such as Vg1 and VegT, that are localized within *X. laevis* oocytes (Zhao et al. 2001). This suggests a potential role in mRNA localization consistent with the ability of human DAZAP1 (hDAZAP1) to shuttle between the nucleus and the cytoplasm (Vera et al. 2002; Lin and Yen 2006). However, a role in mRNA transport/localization/anchoring remains to be firmly established.

A potential role in mRNA translation was initially suggested by the association of hDAZAP1 with the DAZ family of proteins (Tsui et al. 2000a). This family (DAZ, DAZ-like [DAZL], and BOULE) activates the translation of specific mRNAs in metazoan germ cells (Maegawa et al. 2002; Collier et al. 2005; Reynolds et al. 2005, 2007; Brook et al. 2009) via an interaction with the translation factor poly(A)-binding protein (PABP) (Collier et al. 2005). DAZAP1 interacts with the DAZ motif (Maegawa et al. 2002), which is required both for the polysomal association of members of this family and for their ability to fully stimulate translation of reporter mRNAs (Tsui et al. 2000b; Maegawa et al. 2002; Collier et al. 2005). Thus, DAZAP1 could contribute to DAZ-mediated translational activation. Alternatively, it may negatively regulate translation by preventing DAZ binding to proteins, such as PABP, that are required for its ability to activate translation. This is consistent with the observation that human DAZ cannot simultaneously interact in vitro with human PABP1 and DAZAP1 (Morton et al. 2006) and with the absence of Dazap1 on the polysomes in the testis of adult mice (Dai et al. 2001). Thus, although no role in translation has been established for DAZAP1, these studies raise the possibility that DAZAP1 may repress or activate translation and/or modulate the activity of the DAZ family. Since DAZAP1 is expressed in a much wider range of cell types than DAZ family members (Tsui et al. 2000a,b; Dai et al. 2001; Kurihara et al. 2004; Hori et al. 2005; Pan et al. 2005; Brook et al. 2009), DAZAP1 may also regulate translation independently of DAZ proteins, consistent with the ability of many RNA-binding proteins to participate in multiple regulatory complexes. In keeping with this idea, *X. laevis* DAZL (Xdazl) has not been reported to bind Vg1 and VegT mRNAs, suggesting that the roles of DAZAP1 and the DAZ family may be separable even when present in the same cell type.

Here we investigate the attractive hypothesis that DAZAP1 functions as a translational regulator and find that *X. laevis* DAZAP1 can activate translation in an mRNA-specific manner. This activity requires the C terminus of the protein and appears to be regulated during *X. laevis* development, in an ERK-independent manner. In exploring the mechanism of DAZAP1-mediated activation, we find that it stimulates an early step in initiation, downstream from the initial cap-binding event, and present a model in which it regulates end-to-end complex formation independently of a direct interaction with eukaryotic initiation factor (eIF)4G. Similar results were obtained with hDAZAP1 suggesting that this represents an evolutionarily conserved function of DAZAP1 proteins, which may contribute to their critical roles in development and gametogenesis.

RESULTS

X. laevis DAZAP1 stimulates the translation of reporter mRNAs

The interacting protein partners of DAZAP1 suggest potential roles in regulating mRNA utilization in the cytoplasm, including translational control. Tethered-function analysis enables the assessment of a potential role of DAZAP1 in mRNA translation in intact cells without knowledge of its endogenous target mRNAs (Coller et al. 1998; Gray et al. 2000). A luciferase reporter mRNA (luc-MS2) containing 3′ UTR binding sites for the phage MS2 coat protein, a small RNA-binding protein, was co-injected with an internal control mRNA into *X. laevis* oocytes expressing DAZAP1 or control proteins fused to MS2 (Fig. 1). The interaction of MS2 with its cognate RNA-binding sites on the mRNA "tethers" DAZAP1 to the reporter mRNA (Gray et al. 2000), and effects on translation are determined by luciferase assay. Since reporter mRNAs are directly injected into the cytoplasm, any effects on transcription, splicing, or export are negated. Interestingly, luciferase expression (normalized for the activity of the β-galactosidase internal control mRNA) was significantly enhanced (5.5-fold) (Fig. 1A) in oocytes expressing MS2-xDAZAP1 compared with MS2-U1A (Fig. 1A,B). U1A is an RNA-binding protein involved in mRNA splicing (Jovine et al. 1996) rather than translation and acts as a negative control (Gray et al. 2000). Reporter mRNA levels immediately after injection and at the end of the assay period were compared by quantitative RT-PCR analysis. No significant changes were detected, either between oocytes expressing MS2-U1A or MS2-xDAZAP1 or over time (Fig. 1C). This confirms that xDAZAP1 does not affect mRNA stability but rather activates mRNA translation, consistent with the absence of mRNA turnover in stage VI oocytes. Interestingly, the effect of xDAZAP1 is mRNA specific since neither the translation of the control β-galactosidase (data not shown) nor a luciferase reporter mRNA lacking MS2-binding sites (luc-ΔMS2) was stimulated by MS2-xDAZAP1 (Fig. 1D). Similarly, stimulation required that xDAZAP1 be fused to the MS2 protein, since expression of xDAZAP1 alone did not stimulate reporter mRNA translation (Fig. 1E); tethered PABP1 (Gray et al. 2000) served as a positive control (Fig. 1E). In conclusion, xDAZAP1 activates the translation of mRNAs only when bound, indicative of a role as an mRNA-specific activator, adding to the handful of such regulatory proteins identified (Vende et al. 2000; Gorgoni et al. 2005; Cakmakci et al. 2008; Michlewski et al. 2008).

DAZAP1 expression during *X. laevis* embryogenesis

Our findings suggest that the requirement for Dazap1 for normal development may be due, at least in part, to its ability to stimulate translation of specific mRNAs during development; however, little is known about its developmental expression pattern in any species (Dai et al. 2001). To address this, polyclonal antibodies were raised against xDAZAP1, which is described as a 360-amino-acid protein (Zhao et al. 2001). Our antibodies recognized recombinant xDAZAP1 (data not shown) and closely migrating bands in fully grown stage VI oocytes, in vitro matured oocytes, or unfertilized eggs (Fig. 2A,B). Post-fertilization, xDAZAP1 persisted throughout the early cleavages (Fig. 2B). Zygotic transcription ensues at mid blastula transition (stage 8) and is accompanied by a destruction of maternal mRNAs. Following gastrulation, a small increase in xDAZAP1 levels, expressed from zygotically derived mRNA, is observed by the neural plate stage (stage 14), suggesting a requirement for xDAZAP1 protein (Fig. 2B). Levels continue to rise gradually and more markedly from stage 28 until 33/34, with a significant increase at stage 35/36 when the embryos hatch. High levels of xDAZAP1 are maintained until stage 41 (Fig. 2B), during which time external morphological changes, such as tail elongation, continue and organogenesis progresses (for further information on *X. laevis* embryogenesis, see legend to Fig. 2B).

In many developmental stages, xDAZAP1 appeared to be present in multiple forms. This is consistent with a previous study of oogenesis using a different antibody (Zhao et al. 2001). In this study, the faster migrating form ran equivalently to *Escherichia coli* expressed xDAZAP1, suggesting the possibility of post-translational modification. Since hDAZAP1 is phosphorylated by ERK1/2, the phosphorylation status of xDAZAP1 was investigated in stage VI oocytes and progesterone matured oocytes, as many kinase pathways, including ERK2 (ERK1 is not conserved in *X. laevis*), are activated upon maturation. However, phosphatase treatment of extracts from stage VI or mature oocytes did not resolve both forms into a single species, although ERK was efficiently dephosphorylated (Fig. 2C). This does not exclude the possibility that xDAZAP1 is a phosphoprotein, but suggests that the observed forms represent either phosphorylations that are refractory to alkaline phosphatase treatment, alternative post-translational modifications, or splice variants. Interestingly, splice variants of hDAZAP1 are annotated with an alternative 3′ exon that encodes a 378-amino-acid protein, rather than the previously characterized 407-amino-acid protein. To determine whether alternative splicing may account for the multiple forms observed in *X. laevis*, bioinformatic searches were undertaken using the C-terminal exon of the 407-amino-acid form of hDAZAP1. This identified *X. laevis* cDNA sequences that encode a 405-amino-acid xDAZAP1 protein, with the alternative C terminus showing 75% identity with the 407-amino-acid human protein (Fig. 2D). mRNAs corresponding to both splice forms were detectable by RT-PCR analysis of total RNA in stage VI oocytes (data not shown), and similar splice variants are conserved in *Xenopus tropicalis*. The difference in predicted

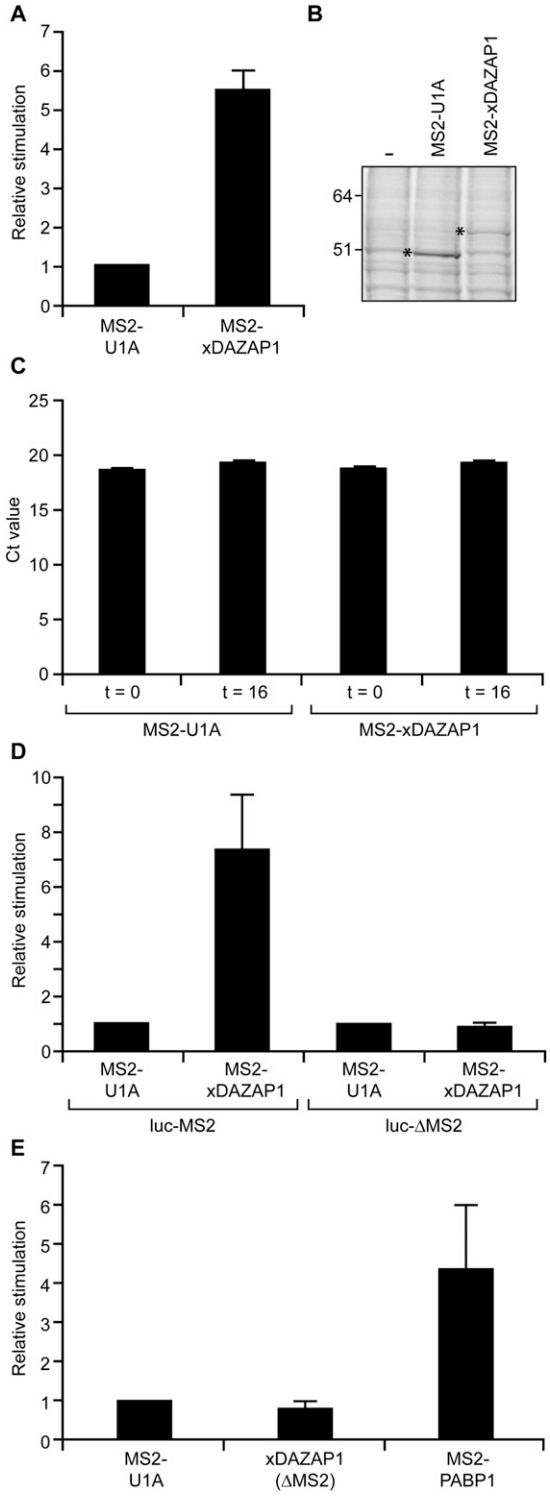

FIGURE 1. (Legend on next page)

molecular weight between the two splice forms is 4 kDa, consistent with the migration of the observed protein bands. Thus both mammalian and *X. laevis* DAZAP1 appear to be expressed as multiple splice variants that vary in their extreme C termini.

xDAZAP1 is present in polysomal fractions

Having established that tethered xDAZAP1 has the capacity to stimulate translation of reporter mRNAs, we tested whether endogenous xDAZAP1 also fulfils this role by investigating its ribosomal association at different developmental stages. To this end, the association of xDAZAP1 with actively translating ribosomes (polysomes) was examined by sucrose gradient analysis of extracts followed by Western blotting of the resulting fractions. Analysis of extracts from stage VI oocytes revealed that xDAZAP1 was essentially restricted to the monosome/diasome fractions of the gradient (Fig. 3A), which contain free 80S ribosomes and mRNAs bound by one or two ribosomes (Gray 1998). This is in contrast to ePABP, a member of PABP family of proteins, that sedimented with both messenger ribonucleoprotein complexes (mRNPs) and polysomes (Fig. 3A, lower panel; Wilkie et al. 2005). This suggests that xDAZAP1 is not associated with efficiently translated mRNAs at this stage, and its unusual sedimentation may be due to its association with large complexes that are not ribosomal. In contrast, in stage 40–41 embryos where xDAZAP1 is more highly expressed, besides being present in fractions containing mRNPs, a significant proportion of xDAZAP1 was found in predominantly lighter polysome fractions (Fig. 3B,C). As expected, PABP1 sedimented with mRNPs and polysomes. Pretreatment of extracts with EDTA, which dissociates RNA–protein complexes including polysomes, caused both xDAZAP1 and PABP1 to largely redistribute to lighter fractions (Fig. 3B), consistent with the association of a proportion of xDAZAP1 with actively translating mRNAs. To confirm its polysome association, extracts were treated with puromycin, which acts as a peptide chain terminator by mimicking the 3′ end of aminoacyl-tRNA and specifically promotes the release of mRNAs from ribosomes. Importantly, puromycin treatment results in the redistribution of the majority of xDAZAP1 and PABP1 to the mRNP fractions (Fig. 3C), establishing that the sedimentation of xDAZAP1 into the heavier fractions is due to its association with endogenous mRNAs on actively translating polysomes. Longer exposures showed that the different forms of xDAZAP1 were represented on polysomes (Fig. 3C). Taken together with the results of our reporter assays, these data strongly support a role for xDAZAP1 in the translational activation of specific endogenous mRNAs.

FIGURE 1. *X. laevis* DAZAP1 can stimulate translation. (*A*) Oocytes expressing MS2-xDAZAP1 or MS2-U1A were co-injected with luc-MS2 reporter and β-galactosidase control mRNAs. Luciferase values were corrected for any minor changes in the levels of β-galactosidase and plotted as relative stimulation compared with the control fusion protein MS2-U1A, which is set to 1. (*B*) SDS-PAGE analysis of ^{35}S-Met–labeled uninjected (−) oocytes or those injected with MS2-U1A or MS2-xDAZAP1 mRNAs. MS2-U1A and MS2-xDAZAP1 contain 21 and 13 methionine residues, respectively. The position of fusion proteins are indicated by asterisks. (*C*) mRNA was isolated from the oocytes in *A* either immediately after injection (t = 0) or after 16 h (t = 16), and mRNA levels were determined by qRT-PCR. Mean Ct values are shown. (*D*) Oocytes expressing MS2-U1A or MS2-xDAZAP1 were injected with β-galactosidase and luciferase mRNAs with (luc-MS2) or without (luc-ΔMS2) MS2-binding sites. (*E*) luc-MS2 and β-galactosidase mRNAs were injected into oocytes expressing MS2-U1A, ΔMS2-xDAZAP1, or MS2-PABP1, which serves as a positive control. Data in *D* and *E* are plotted as described in *A*.

Translational activation by DAZAP1 is conserved

Our findings with respect to xDAZAP1 differ from the proposed roles for mammalian DAZAP1 (Dai et al. 2001; Morton et al. 2006), although the long and short protein forms are 82% and 83% identical, respectively. However, the role of mammalian DAZAP1 in translation has never been directly tested. To address this, an MS2-hDAZAP1 fusion, containing the previously studied 407-amino-acid (long) form (Morton et al. 2006; Yang et al. 2009), was expressed in stage VI *X. laevis* oocytes (Fig. 4A). Consistent with their predicted molecular weights, MS2-hDAZAP1 showed reduced mobility in SDS-PAGE compared with the 360-amino-acid form of xDAZAP1 in oocytes (cf. Figs. 4A and 1B) or when expressed in vitro (Fig. 4B). Interestingly, injection of the tethered-function reporter mRNAs into oocytes expressing MS2-U1A, MS2-hDAZAP1, or MS2-DAZL as a positive control for translational activation (Collier et al. 2005) revealed that hDAZAP1 was also capable of stimulating translation (Fig. 4C), as mRNA stability was not altered (data not shown). The level of activation by hDAZAP1 was quantitatively similar to that of xDAZAP1 (Fig. 1A), and the effects of hDAZAP1 were also mRNA specific (Fig. 4D), suggesting that this function is an evolutionarily conserved property of both short and long forms of DAZAP1.

ERK phosphorylation does not regulate the translational activity of DAZAP1

ERK1/2 phosphorylation on threonine 269 and 315 (Fig. 2D) can affect the protein interactions mediated by hDAZAP1 in vitro (Morton et al. 2006). These threonine residues are conserved, albeit in an altered context in xDAZAP1 (Fig. 2D), and our polysome analysis (Fig. 3A) supports the possibility that xDAZAP1 has roles in addition to translational control, consistent with the idea that ERK may regulate its participation in translation complexes. Thus we examined the relative ability of xDAZAP1 to stimulate translation in both stage VI and mature oocytes, since ERK2 is activated in the latter (Fig. 2C). In contrast to some other characterized regulators (e.g., Gorgoni et al. 2005; Wilkie et al. 2005), the ability of DAZAP1 to stimulate translation was not significantly

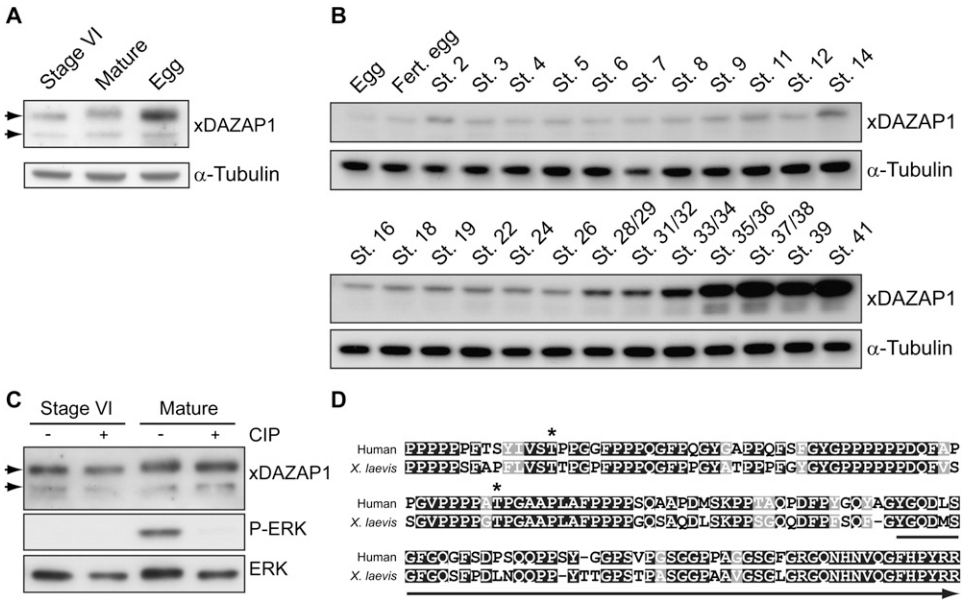

FIGURE 2. Developmental expression pattern and alternative splicing of *X. laevis* DAZAP1. Western blots of extracts probed with antibodies against xDAZAP1 or tubulin (control), prepared from (*A*) fully grown oocytes (stage VI), in vitro matured (mature), or unfertilized eggs (egg); or (*B*) unfertilized eggs or embryos (up to stage 41) according to the tables of Nieuwkoop and Faber (1994). After the 12 initial cleavages following fertilization, zygotic transcription ensues at the mid blastula transition (stage 8) with gastrulation being initiated at stage 10. The neural plate (stage 14) starts to fold at stage 15, with the neural tube being completely closed by stage 21. The tail bud is discernable at stage 24, at which time embryos exhibit response to external stimuli with spontaneous movement beginning at stage 26. By stage 28, the general pattern of the brain is largely established, and by stage 29/30, the tail bud is distinct with 24–25 somites segregated, with segregation reaching the tail. The heart starts beating at stage 33/34 with hatching initiating at stage 35/36, when the length of the tail bud is about three times its breadth. The entire pronephros is functional by stage 37/38, with the mouth breaking through at stage 40 and initial torsion of the intestine and segregation of the primordial germ cells occurring at stage 41. (*C*) Extracts from stage VI and progesterone matured oocytes were untreated (−) or treated with calf intestinal phosphotase (CIP; +) and analyzed by Western blot using the indicated antibodies. ERK2 is active when phosphorylated (P-ERK). (*D*) Comparison of the C-terminal region of the long forms of xDAZAP1 and hDAZAP1, with identities shown in black and similarities shown in gray. The newly identified alternative C-terminal exon of xDAZAP1 is underlined, and ERK-phosphorylated threonine residues in hDAZAP1 are indicated by asterisks.

affected by maturation (Fig. 5A), suggesting that this DAZAP1 activity is not regulated by signaling pathways that are activated upon maturation. However, it is not known whether xDAZAP1 is an in vivo substrate of ERK2 during maturation or whether such phosphorylation is highly transient. Therefore to examine the role of ERK directly, we introduced point mutations that act as phosphomutants (AA) or phosphomimetics (DD) of threonine 269 and 315 in hDAZAP1. Neither manipulation significantly altered hDAZAP1 activity in either stage VI (data not shown) or mature oocytes (Fig. 5B), showing that phosphorylation at these residues does not regulate protein interactions that are required for the translational stimulatory activity of DAZAP1.

Multiple binding sites enhance the level of activation by DAZAP1

Many 3′ UTR-binding proteins bind to elements that are present in multiple copies within the 3′ UTR, with multiple elements having an additive effect on translational regulation (e.g., Ostareck-Lederer et al. 1994; Collier et al. 2005), although others only benefit from a single binding site (Sanchez and Marzluff 2002; Gorgoni et al. 2005). To investigate whether activation by DAZAP1 is sensitive to the number of molecules that can bind to the 3′ UTR, reporter mRNAs with different numbers of MS2-binding sites were tested (Fig. 6). xDAZAP1 weakly stimulated the translation of an mRNA with one binding site (Fig. 6), with the magnitude of stimulation markedly increasing in the presence of three binding sites (Fig. 6). However the translation of a reporter mRNA containing nine, rather than three, binding sites was not further enhanced (Fig. 6). hDAZAP1 showed similar functional characteristics (Supplemental Fig. S1). This suggests that the physiological target mRNAs that are translationally regulated by DAZAP1, including those that may be required for normal development, may contain multiple DAZAP1-binding sites.

DAZAP1 enhances translation initiation

To begin to address the mechanism by which DAZAP1 activates translation, we sought to determine whether it acts during initiation or later, during elongation or termination. To this end, we utilized a nonphysiologically capped reporter mRNA containing a classical swine fever virus (CSFV) internal ribosome entry site (IRES) within its 5′ UTR (Fig.

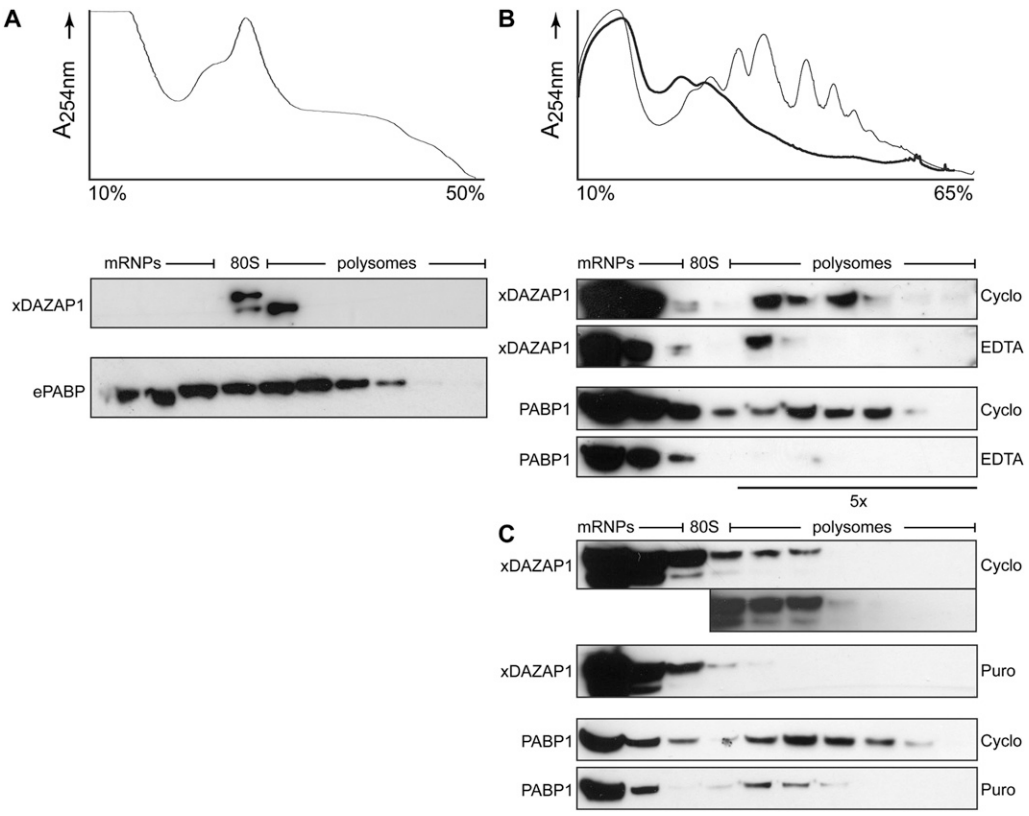

FIGURE 3. Polysomal association of *X. laevis* DAZAP1. Stage VI *X. laevis* oocytes (*A*) or stage 40–41 embryos (*B*,*C*) were subjected to sucrose gradient analysis, and the resulting UV absorbance profiles (254 nm) of cycloheximide-(Cyclo, thin line) or EDTA-treated (thick line) extracts are shown. In stage VI oocytes, polysome peaks are not observed, as only 2% of ribosomes are actively engaged in translation, with the majority of ribosome subunits associating as empty 80S couples. By stage 41, polysome peaks can be clearly discerned. The resulting fractions were probed by Western blot for xDAZAP1 and either ePABP (*A*) or PABP1 (*B*,*C*), depending on developmental stage. EDTA (*B*) and puromycin (puro; *C*) treatment both cause the majority of xDAZAP1 and PABP1 to be released from polysomes. In *B*, five times more protein was loaded into fractions 5–10 to allow enhanced visualization of the association of DAZAP1 and PABP1 with polysomes. The longer exposure of polysomal fractions in *C* shows that both forms of xDAZAP1 associate with polysomes. The positions of polysomes, the 80S monosome, and mRNP fractions are indicated.

7A; Supplemental Fig. S2A). Translation is initiated at IRESs via an alternative pathway through which 40S ribosomal subunits are directly recruited to a site within the 5′ UTR independently of the 5′ cap (Jackson 2005; Doudna and Sarnow 2007). Although the CSFV IRES directs efficient translation in oocytes (Gorgoni et al. 2005), neither xDAZAP1 (Fig. 7A) nor hDAZAP1 (Supplemental Fig. S2A) was able to stimulate translation driven by this IRES, although they activated cap-dependent translation (Fig. 7A; Supplemental Fig. S2A). Since elongation and termination, but not initiation, progress normally in CSFV IRES-mediated translation (Pestova et al. 1998, 2008), this reveals that DAZAP1 acts during the initiation phase of translation. Initiation has several mRNA-dependent steps (Pestova et al. 2007). The cap is bound by the eIF4F complex, which consists of the cap-binding factor eIF4E, the scaffold protein eIF4G, and eIF4A, an RNA helicase whose activity in removing secondary structure within the 5′ UTR is stimulated by eIF4B. Subsequent binding of the small ribosomal subunit and associated factors (eIF2-GTP-Met, eIF3, eIF1, eIF1A, eIF5) is aided by eIF4G–eIF3 interactions. This complex then scans the 5′ UTR for an initiation codon, and upon its recognition, initiation factors are released and the large ribosomal subunit joins. As CSFV only deviates from the cap-dependent pathway during the earlier steps of initiation (Pestova et al. 1998, 2008), this suggests that DAZAP1 acts prior to large ribosomal subunit recruitment.

DAZAP1 can stimulate translation independently of the cap

IRESs differ significantly in their mechanism of ribosomal subunit recruitment and initiation factor requirements, making them a useful tool with which to dissect initiation mechanisms (Jackson 2005; Doudna and Sarnow 2007). The inability of DAZAP1 to stimulate initiation from a CSFV IRES led us to question whether DAZAP1 activity is strictly cap-dependent and, therefore, incapable of stimulating cap-independent translation in general. To address this, we examined the ability of DAZAP1 to stimulate mRNAs that

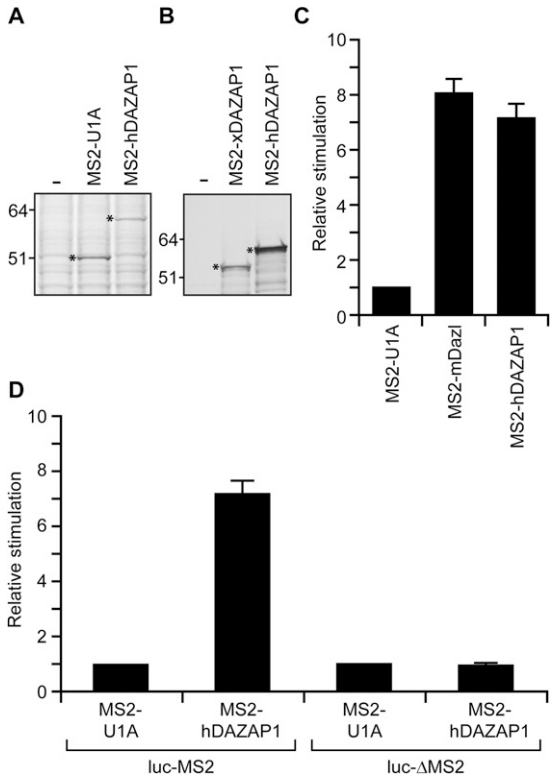

FIGURE 4. Human DAZAP1 has a conserved ability to stimulate translation. SDS-PAGE analysis of (*A*) ^{35}S-Met labeled uninjected (−) oocytes or those injected with MS2-U1A or MS2-hDAZAP1 mRNAs or (*B*) in vitro translated MS2-xDAZAP1, MS2-hDAZAP1, or control (−). MS2-U1A, MS2-hDAZAP1, and MS2-xDAZAP1 contain 21, 12, and 13 methionine residues, respectively. The positions of fusion proteins are indicated by asterisks. (*C*) Tethered-function analysis in oocytes expressing MS2-U1A, MS2-hDAZAP1, or MS2-mouse Dazl (mDazl), an mRNA-specific activator that serves as a positive control. (*D*) Oocytes expressing MS2-U1A or MS2-hDAZAP1 were injected with luciferase mRNAs with (luc-MS2) or without (luc-ΔMS2) MS2-binding sites and a β-galactosidase mRNA. Data are plotted as in Figure 1A.

contain an alternative IRES or possess an ApppG cap and are translated in a non-IRES–mediated cap-independent manner (Fig. 7B,C; Supplemental Fig. S2B,C). The hepatitis A virus (HAV) IRES, which can promote cap-independent initiation in *X. laevis* oocytes (RWP Smith, O Larralde, B Gorgoni, P Malik, SV Graham, and NK Gray, in prep.), differs from the CSFV IRES as it requires all of the canonical initiation factors, including the cap-binding factor eIF4E, which is not required by the majority of characterized IRESs (Jackson 2005). However, eIF4E is not involved in cap recognition in HAV IRES-mediated initiation but rather induces a translation-competent conformation in eIF4G bound to the IRES (Ali et al. 2001; Borman et al. 2001). The translation of ApppG-capped mRNAs occurs independently of eIF4E, which specifically recognizes m^{7}GpppN (Pestova et al. 2007), and with dramatically reduced efficiency in oocytes (Gillian-Daniel et al. 1998), in which translation is highly cap-dependent. Injection of the HAV IRES or cap-independent reporter mRNA into oocytes expressing either MS2-xDAZAP1 (Fig. 7B,C) or MS2-hDAZAP1 (Supplemental Fig. S2B,C) fusion proteins showed that these stimulated translation of both reporter mRNAs. The HAV IRES and cap-independent reporters were stimulated with somewhat reduced or enhanced efficiency, respectively, compared with the cap-dependent mRNA; the former may be a result of noncanonical use of eIFs. Thus, DAZAP1-mediated regulation is not strictly cap-dependent, and our data indicate that DAZAP1 does not act to enhance binding of the cap by the eIF4F complex, via eIF4E, but acts to enhance initiation downstream from this event.

DAZAP1-mediated regulation is poly(A)-sensitive

Translation initiation is promoted by the poly(A) tail, which like the 5′ cap is a primary determinant of translational efficiency (Kahvejian et al. 2005) through its role in forming so-called end-to-end complexes or closed-loop conformations, which bring the 5′ and 3′ UTRs into proximity via an interaction between PABP, bound to the poly(A) tail, and eIF4G bound to the 5′ UTR as part of the eIF4F complex. Poly(A) tail length is dynamic and highly regulated during gametogenesis and development (Richter 1999), and thus, we sought to determine whether the activity of DAZAP1 could be affected by the adenylation status of target mRNAs by using adenylated and nonadenylated reporter mRNAs in tethered-function assays (Fig. 8; Supplemental Fig. S3). As expected, polyadenylated mRNAs were translated more

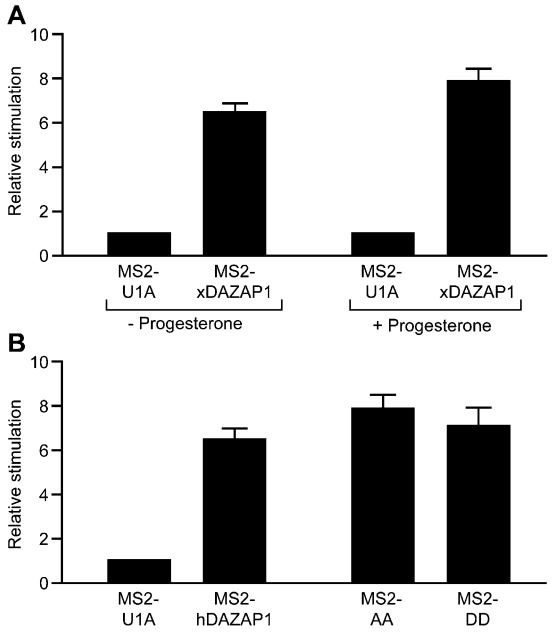

FIGURE 5. ERK phosphorylation does not regulate the translational stimulatory activity of DAZAP1. Tethered-function assays in stage VI (*A*) and mature oocytes (*A*,*B*) expressing (*A*) MS2-xDAZAP1 or (*B*) wild-type MS2-hDAZAP1 or MS2-hDAZAP1 containing phosphomutant (AA) or phosphomimetic residues (DD). Reporter and internal control mRNAs were used, and the data are plotted as in Figure 1A.

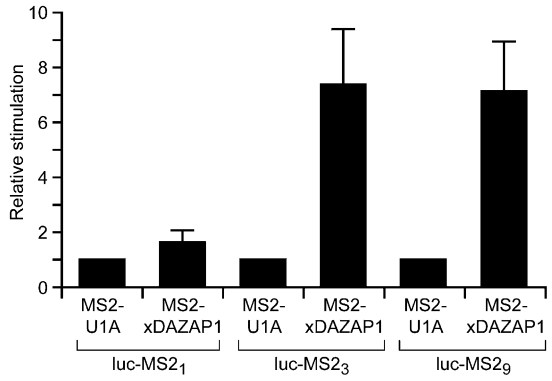

FIGURE 6. DAZAP1-mediated stimulation is enhanced by the presence of multiple binding sites within the 3′ UTR. Oocytes expressing MS2-U1A and MS2-xDAZAP1 were co-injected with β-galactosidase control and luciferase reporter mRNAs containing one (luc-MS2$_1$), three (luc-MS2$_3$), or nine (luc-MS2$_9$) MS2-binding sites within the 3′ UTR. luc-MS2$_3$ is referred to as luc-MS2 elsewhere. Data are plotted as in Figure 1A.

efficiently than their nonadenylated counterparts, but importantly, polyadenylated mRNAs bound by either xDAZAP1 or hDAZAP1 were translated most efficiently (Fig. 8A; Supplemental Fig. S3A). However, the relative stimulation by either DAZAP1 was reduced by the presence of a long poly(A) tail (Fig. 8B; Supplemental Fig. S3B), suggesting that DAZAP1 and the poly(A) tail both contribute to initiation by promoting the formation of end-to-end complexes.

The domains required for translational stimulation do not interact directly with eIF4G

To explore the regions of DAZAP1 required to stimulate initiation, a series of deletion mutants were created that remove one or both of the RRMs or the C-terminal region (Fig. 9A). Tethering allows the activation capacity of domains to be assessed independently of their role in RNA binding, as this function is provided by the MS2-fusion protein. Deletion of the proline-rich C terminus (MS2-ΔC) dramatically reduced stimulation to just 27% of full-length xDAZAP1 activity (Fig. 9B). The C-terminal region alone (MS2-Δ1+2) stimulated translation to 46% of the full-length level, whereas RRM2 plus the C-terminal region (MS2-Δ1) conferred 77% stimulatory activity (Fig. 9B). This suggests that the translational activation function of DAZAP1 is mainly conferred by a region that contains RRM2 and at least part of the C terminus.

Interestingly, a previous study with GST-DAZAP1 transfected into HEK-293 cells identified a large number of RNA-binding proteins, including a single translation factor, eIF4G, as potential partners of DAZAP1 (Yang et al. 2009). Deletion of RRM1 and part of RRM2 of DAZAP1 greatly reduced the affinity of eIF4G for DAZAP1 (Yang et al. 2009). Taken together with our findings that a region containing RRM2 appears to be important for DAZAP1 activity, this raises the attractive hypothesis that eIF4G may be a direct protein partner of DAZAP1. To test this, we undertook a yeast two-hybrid approach. Neither human nor *X. laevis* DAZAP1 interacted with an N-terminal region of eIF4G, nor did they interact with eIF4G lacking only the extreme N terminus that contains the PABP1-binding site (Fig. 9C; Pestova et al. 2007). As expected, eIF4E interacted with both eIF4G fragments. To confirm the absence of an interaction between eIF4G and DAZAP1, pull-downs with purified

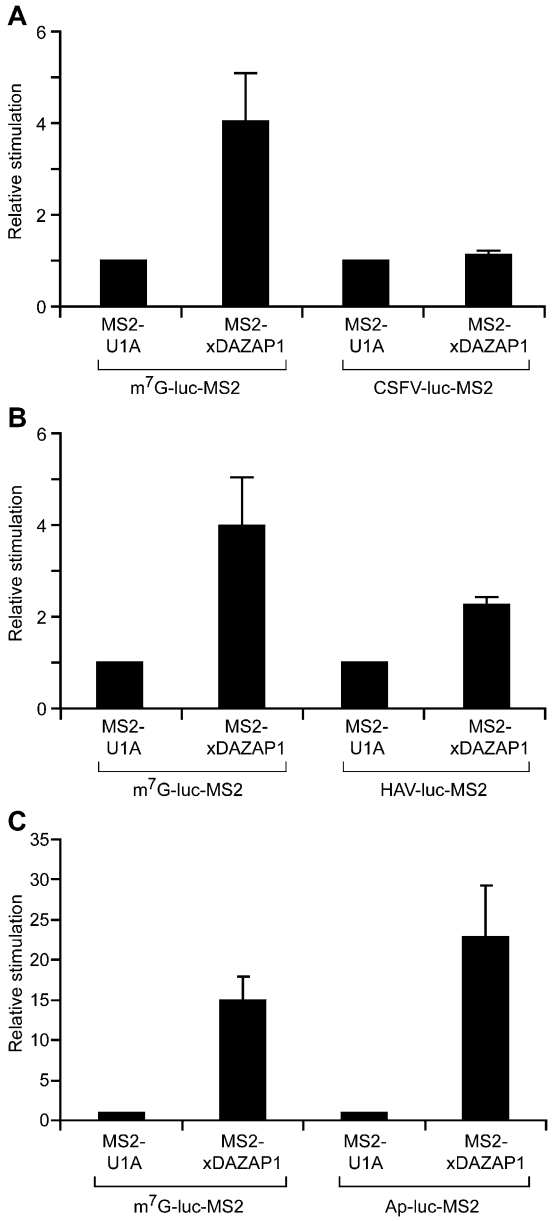

FIGURE 7. DAZAP1 can stimulate translation initiation in a cap-independent manner. Oocytes expressing MS2-U1A and MS2-xDAZAP1 were co-injected with β-galactosidase control mRNA and either m^{7}G-capped luc-MS2, CSFV-luc-MS2 (*A*), HAV-luc-MS2 (*B*), or ApG-capped-luc-MS2 (*C*) mRNA. Data are plotted as in Figure 1A.

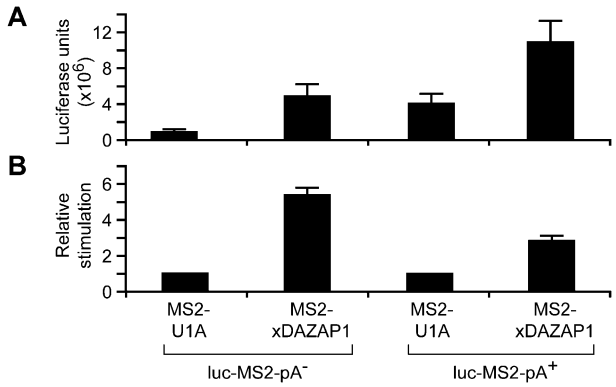

FIGURE 8. DAZAP1-mediated stimulation is sensitive to the presence of the 3′ poly(A) tail. Oocytes expressing MS2-U1A and MS2-xDAZAP1 were co-injected with luc-MS2 (luc-MS2-pA$^-$) and luc-MS2-pA and the β-galactosidase control mRNA. (*A*) Raw luciferase values corrected for β-galactosidase. (*B*) Relative stimulation with MS2-U1A set to 1.

recombinant proteins expressed in *E. coli* were undertaken (Fig. 9D). Flag-eIF4G failed to pull down DAZAP1 but efficiently pulled down PABP1, a known partner protein (Fig. 9D, lower panel). Thus, a direct interaction between eIF4G and DAZAP1 does not appear to underlie the ability of DAZAP1 to stimulate translation when bound to the 3′ end of mRNAs.

DISCUSSION

Our results support a conserved role for both short and long forms of DAZAP1 in mRNA-specific translational activation. We find that xDAZAP1 is associated with polysomes (Fig. 3), and show that it can robustly stimulate the translation of reporter mRNAs (Fig. 1). These observations appear at odds with the suggestion that the long form of hDAZAP1 represses translation (Morton et al. 2006). However its role in translation was not previously addressed experimentally, and we show that mammalian DAZAP1 efficiently activates translation (Fig. 4). Importantly, we find that DAZAP1 only stimulates the translation of mRNAs to which it is bound (Figs. 1, 4), showing that it does not act as a global regulator of initiation, expanding the relatively small number of mRNA-specific activators that have been described to date.

In most cases of mRNA-specific regulation, and particularly translational activation, the underlying mechanisms are not well defined. Our results provide an initial insight into the mechanism by which DAZAP1 stimulates translation. By using an mRNA dependent for its translation on the CSFV IRES, we were able to show that DAZAP1 regulates translation initiation (Fig. 7; Supplemental Fig. S2). As events prior to and including small ribosomal subunit joining are noncanonical during CSFV-driven initiation, our results suggest that an early step in initiation is regulated by DAZAP1. However, the ability of DAZAP1 to direct HAV IRES-mediated and

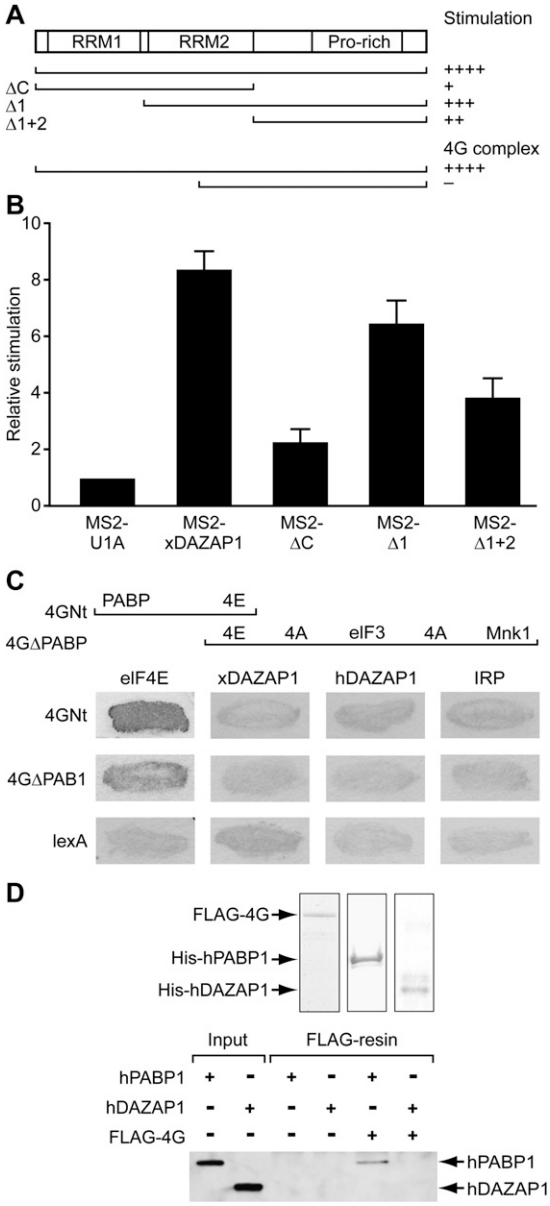

FIGURE 9. DAZAP1 C-terminal regions stimulate translation independently of a direct interaction with eIF4G. (*A*) Cartoon showing the constructs utilized in *B*, summarizing their relative ability to stimulate translation, and the co-isolation of eIF4G with full-length but not truncated GST-DAZAP1 in Yang et al. (2009). (*B*) Oocytes expressing MS2-U1A, MS2-xDAZAP1, MS2-ΔC (amino acids 1–192), MS2-Δ1 (amino acids 106–360), or MS2-Δ1+2 (amino acids 193–360) were co-injected with the luc-MS2 reporter and β-galactosidase control mRNAs. Data are plotted as in Figure 1A. (*C*, *top*) Cartoon showing the eIF4G truncations used and the relative locations of binding sites for the indicated partner proteins. (*Bottom*) Yeast two-hybrid assay using 4GNt or 4GΔPABP or MS2 (negative control; indicated by lexA) fused to the LexA-DNA-binding domain and xDAZAP1, hDAZAP1, eIF4E (positive control), or IRP (iron-responsive protein, negative control) fused to the GAL4-activation domain. Interactions were detected by qualitative β-galactosidase filter assays. (*D*, *top*) SDS-PAGE analysis of the indicated purified recombinant proteins visualized with Gelcode Blue. (*Bottom*) Pull-down of Flag-tagged eIF4G (FLAG-4G) in the presence of purified His-tagged PABP1 or DAZAP1 analyzed by Western blot using anti-His antibodies.

cap-independent translation (Fig. 7; Supplemental Fig. S2) shows that its activity is not strictly dependent on the initial cap-recognition event. Thus, DAZAP1 appears to function downstream from cap binding, distinguishing its activity from other characterized 3′ UTR–bound activators that target this step (Vende et al. 2000; Gorgoni et al. 2005; Cakmakci et al. 2008; Michlewski et al. 2008).

Although DAZAP1-mediated stimulation does not require the presence of a poly(A) tail, it is sensitive to its presence (Fig. 8; Supplemental Fig. S3). DAZAP1 has some homology with Musashi (Akindahunsi et al. 2005), a protein that can repress translation but also promotes cytoplasmic polyadenylation (Charlesworth et al. 2006), raising the possibility that DAZAP1 may stimulate initiation indirectly by promoting polyadenylation. However, the reporter assays were performed in stage VI oocytes prior to the onset of cytoplasmic polyadenylation, and a required sequence element (A_2UA_3) (Richter 2007) was not present in the reporter mRNAs, making it highly unlikely that changes in poly(A) tail length underlie the observed effects.

The activity of the poly(A) tail in promoting initiation is mediated by PABP, which interacts with eIF4G and other initiation factors bound to the 5′end of mRNAs, forming end-to-end complexes (Mangus et al. 2003). The decrease in relative stimulation in the presence of the poly(A) tail (Fig. 8; Supplemental Fig. S3) raises the possibility that DAZAP1 may play a role in regulating the formation of such complexes (Fig. 10). In germ cells, but not in other cells, this could be mediated by DAZ family proteins since these interact with both DAZAP1 and PABP1. However, triple DAZAP1–DAZ–PABP complexes fail to form in vitro, and the interaction of DAZ family proteins with DAZAP1 or PABP appears mutually exclusive (Morton et al. 2006). Moreover, the absence of an effect of mutations that disrupt the DAZAP1–DAZ interaction (Morton et al. 2006) suggests that interactions with this family are not required for DAZAP1 to stimulate translation (Fig. 5). It remains a possibility that other 3′ UTR–binding proteins may mediate an indirect interaction with PABP (Fig. 10). Alternatively, DAZAP1 may stabilize PABP-eIF4G–mediated complexes or participate in alternative end-to-end complexes by forming additional protein–protein contacts between the 5′ and 3′ UTRs (Fig. 10). Such alternative complexes have been described in the activation of histone mRNA translation during oocyte maturation (Cakmakci et al. 2008). This may be achieved by direct or indirect interactions of DAZAP1 with translation initiation factors bound to the 5′ UTR. Intriguingly, eIF4G was shown to be in complexes with DAZAP1 (Yang et al. 2009), an association that requires RRM2, a region that is important for stimulatory activity (Fig. 9). A role of eIF4G is also consistent with our findings that DAZAP1 can promote cap-dependent, cap-independent, and IRES-mediated initiation (Fig. 7; Supplemental Fig. S2), functions that are mediated in part by the ability of eIF4G to bind RNA (Yanagiya et al. 2009). However, eIF4G and DAZAP1 do not appear to directly interact (Fig. 9), indicating that any effect on eIF4G function is indirect.

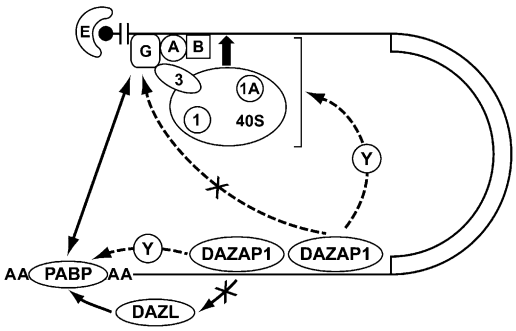

FIGURE 10. A model for DAZAP1-mediated translational activation. DAZAP1 stimulates initiation by participating in or stabilizing end-to-end complex formation, which functionally links the 5′ and 3′ ends of the mRNA, enhancing small ribosomal subunit recruitment (filled arrow). Our results show that DAZAP1 can mediate this effect independently of the initial cap-binding step (denoted by broken line). DAZAP1 does not utilize Xdazl (denoted by a cross), but other proteins may mediate contact between DAZAP1 and PABP (factor Y), stabilizing complexes formed between PABP and eIF4G. Alternatively, DAZAP1 may contact factors at the 5′ end providing additional end-to-end contacts that help stabilize the closed-loop conformation. Since CSFV and the HAV IRES differ in their requirement for eIF4G, eIF4A, eIF4B, eIF1, or eIF1A, one of these 5′-bound factors may be contacted directly or indirectly (via factor Y) by DAZAP1; other eIFs are omitted for clarity. Direct interactions with eIF4G do not occur (denoted by a cross). Multiple DAZAP1 molecules are shown to indicate that target mRNAs benefit from multiple binding sites. Solid and dotted arrows indicate known and potential interactions, respectively. eIFs are denoted by their numbers or, with eIF4 factors, their letters: AAAA, poly(A) tail; Y, hypothetical factor(s); 40S, small ribosomal subunit; solid circle, 5′ cap; and curved box, open reading frame.

Interestingly, we find that the expression of DAZAP1 and its association with polysomes is developmentally controlled (Figs. 2, 3), the latter suggesting that its role in activating translation may be subject to regulation. However, the use of phosphomimetic and phosphomutant forms of hDAZAP1 appears to rule out a role of phosphorylation of Thr269 or 315 in this process. The regulated polysome association of xDAZAP1 (Fig. 3) and our results with tethered hDAZAP1 (Fig. 4) suggest that analysis at particular stages of spermatogenesis or development may reveal polysomal association of mammalian Dazap1, which was not observed in adult mouse testis (Dai et al. 2001).

xDAZAP1 from stage VI oocytes sediments to the monosome/diasome region of the gradient (Fig. 3A). This may reflect an association with mRNAs that are being actively translated by only a small number of ribosomes, the presence of stalled ribosomes (Wang et al. 1998), or large protein complexes that block translation (e.g., Gray and Hentze 1994) and/or direct mRNA localization (Gavis et al. 2007). There is substantial precedent for multi-functional RNA-binding proteins that link translational activation and repression, mRNA localization, or stability (Gray and Wickens 1998), and our data (Fig. 3) combined with that of Zhao et al. (2001) support a developmental switch in xDAZAP1 activity

from predominantly associating with localized mRNAs to promoting translation. Thus we favor a model in which DAZAP1 participates in multiple gene regulatory events and may, in some cases, exit the nucleus bound to mRNAs, directing their subsequent fate in the cytoplasm. Both phosphorylation (Morton et al. 2006) and alternative splicing (Fig. 2) may control the association of DAZAP1 with complexes that are important for functions other than translational activation.

Consistent with our finding that DAZAP1 stimulates translation in an mRNA-specific manner (Figs. 1, 4), there is evidence that DAZAP1 is an mRNA-specific binding protein in a variety of species. It was identified as an AU-rich binding protein by RNA-affinity chromatography (Morton et al. 2006), shown to have a preference for AAAUAG and $GU_{1-3}AG$ sequences by in vitro RNA selection (Hori et al. 2005), and isolated in association with the 3′ UTR of specific localized mRNAs (Zhao et al. 2001) or RNAs containing specific splice sites (Goina et al. 2008; Skoko et al. 2008). Our findings suggest that mRNAs that are translationally activated by DAZAP1 may contain multiple DAZAP1-binding sites within their 3′ UTRs (Fig. 6; Supplemental Fig. S1); however, verification of this will require the identification of endogenous DAZAP1 target mRNAs. Since DAZAP1 appears to be multifunctional, the identification of mRNAs whose translational activation by DAZAP1 underlies its contribution to development is complicated by the likelihood that only a subset of DAZAP1-bound mRNAs will be translationally controlled by DAZAP1. Therefore, translational profiling in knockout or knockdown animals will be required to identify these mRNAs. This will first require a more detailed description of the developmental defects associated with loss of function to identify which developmental stages and cell types may contain mRNAs that are translationally misregulated in the absence of DAZAP1.

In summary, this study identifies DAZAP1 as a novel regulator of mRNA-specific translation that acts downstream from the initial cap-binding event and raises interesting questions pertaining to its mechanism of action. Thus, future work will be directed at identifying direct or indirect interactions of DAZAP1 with translation factors bound to the 5′ or 3′ ends of the mRNA.

MATERIALS AND METHODS

Plasmids

Plasmids expressing MS2-fusion proteins pMS2-U1A, pMS2-PABP (Gray et al. 2000), pMS2-Dazl (Collier et al. 2005), and pMSPN (MS2) (Wilkie et al. 2005) have been previously described. Plasmids expressing reporter mRNAs pLG-MS2 (luc-MS2, also known as luc-$MS2_3$), pLGENB1 (luc-ΔMS2) (Gray et al. 2000), pMS2-1, pMS2-9, pLuc-MS2-pA (Collier et al. 2005), pPV-Luc-MS2, pCSFV-Luc-MS2 (Gorgoni et al. 2005), pJK350 (β-galactosidase) (Evans et al. 1994), pHAV-Luc-MS2, and pCSFV-*lacZ* (RWP Smith, O Larralde, B Gorgoni, P Malik, SV Graham, and NK Gray, in prep.) have been described. pMS2-hDAZAP (containing DAZAP1-201 [ENST00000233078]) and the AA and DD derivatives were generated by Mark W. Peggie and were a gift from Philip Cohen. pMS2-Prrp, containing xDAZAP1 was created from pET-Prrp (a gift from Paul Huber) by PCR using primers

5′-GTCAGTGTCAGTGCTAGCATGAACAACCAAGGCGGGGAC-3′
and
5′-GTCAGTACTAGTTCAAATCCACTCGGACAATTTCAC-3′

and inserted into pMSPN after digestion with NheI and SpeI. pMSPN-ΔC (encoding an MS2 fusion of xDAZAP1 amino acids 1–192), pMSPN-Δ1 (MS2-xDAZAP1 amino acids 106–360), and pMSPN-Δ1+2 (MS2-xDAZAP1 amino acids 193–360) were created by PCR amplification of xDAZAP with primer pairs

5′-GTCAGTGTCAGTGCTAGCATGAACAACCAAGGCGGGGAC-3′ and
5′-CGCACTAGTTCATGGTTCTGCCCGTTTGACTTC-3′,
5′-GCGCGCTAGCCCCAGAACTGAAAACAGTAGGTC-3′ and
5′-GTCAGTACTAGTTCAAATCCACTCGGACAATTTCAC-3′,

and

5′-AGCTGCTAGCCGTGATAGCAAAAGCCAAACTCCAGG-3′
and
5′-GTCAGTACTAGTTCAAATCCACTCGGACAATTTCAC-3′,

respectively, and insertion of the resulting products into the NheI and SpeI sites in pMSPN. Plasmids used for yeast two-hybrid analysis pBTM-4GNt, LexA-MS2, pACT-IRP (Gray et al. 2000), pACT-eIF4E, and pBTM-eIF4G1 (expressing 4GΔPABP) (Gorgoni et al. 2005) were previously described. Plasmids for expression of GAL4 activation domain fusions of hDAZAP1 (pACT-hDAZAP1) and xDAZAP1 (pACT-xDAZAP1) were created by PCR amplification of hDAZAP1 and xDAZAP1 with primer pairs

5′-GTGTCAGTCCATGGAGATGAACAACTCGGGCGCCGAC-3′
and
5′-GTGTCAGTGGATCCCTAGCGTCGGTAGGGGTGGAACCC-3′,

and

5′-GTCAGTGTCAGTCCATGGAGATGAACAACCAAGGCGGGGAC-3′ and
5′-GTGTCAGTAGATCTTCAAATCCACTCGGACAATTTCAC-3′,

respectively, and insertion of the resulting products into the NcoI and BamHI sites of pACT2 (Clontech). pET-hPABP1 was created by PCR of hPABP1 with primer pair

5′-GGATCCTTATGAACCCCAGTGCCCC-3′ and
5′-GTCGACTCAAACAGTTGGAACACCGGTGG-3′

and insertion of the product into BamH1 and Sal1 sites of pET-28c(+) (Novagen).

Isolation of *X. laevis* oocytes and generation of embryos

Oocytes were manually dissected or released by collagenase treatment—2.5 mg/mL collagenase in Marc's modified ringer's

solution (MMR; 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES at pH 7.4, and 1 μg/mL of penicillin and streptomycin) for 1 h 30 min—from excised portions of ovary and incubated at 18°C–22°C in MMR. Oocytes were matured by the addition of 10 mg/mL progesterone (Sigma) and scored by the appearance of a white spot on the animal pole. To obtain eggs for fertilization, adult females were injected with 50 U of pregnant mare serum (Calbiochem) 2–5 d prior to oviposition and induced to lay eggs by injection with 600 U of human chorionic gonadotropin hormone (Sigma). Eggs were collected and smeared with testes, macerated in 0.5× MMR. Fertilized eggs were identified by contraction of the animal hemisphere and cortical rotation, de-jellied in 2% cysteine (pH 8.0), and subsequently reared in 0.1× MMR. Embryos were staged according to the tables of Nieuwkoop and Faber (1994) and collected by freezing on dry ice.

Tethered-function analysis and metabolic labeling

Tethered-function assays were performed as described previously (Gray et al. 2000). Briefly, in vitro transcribed mRNAs encoding MS2-fusion proteins were microinjected into stage VI *X. laevis* oocytes. Following incubation, reporter and control mRNAs were co-injected, and oocytes were incubated overnight. Luciferase reporter mRNAs were m^7GpppG-capped except those containing an IRES or when specifically stated (ApppG). At least three groups of five oocytes were harvested per point, and the levels of luciferase and β-galactosidase activity were assayed in duplicate. Data are derived from a minimum of three experimental repeats, and error bars on the graphs represent SEM. Expression of MS2-fusion proteins was confirmed by metabolic labeling with 100 μCi/mL [^{35}S]-methionine for 6 h, as described (Gray et al. 2000).

In vitro protein expression

MS2-xDAZAP1 and MS2-hDAZAP1 were expressed from pMS2-Prrp and pMS2-hDAZAP, respectively, using the TNT/T7 system (Promega) supplemented with 10 μCi ^{35}S-methionine.

RNA stability analysis

Total RNA was extracted from injected oocytes (Gray et al. 2000), and first-strand cDNA was synthesized from total RNA with an AMV Reverse Transcriptase Kit (Roche) according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed with an ABI 7500 Fast Real-Time PCR System (ABI) by SYBR I green incorporation with luciferase primers

5′-GGCGCGGTCGGTAAAGTT-3′ and
5′-AGCGTTTTCCCGGTATCCA-3′.

Data analysis was performed with AB17500 software. All samples were analyzed in duplicate, and the mean Ct values were plotted. Error bars represent SEM.

Polysome analysis

Three milligrams of stage VI oocyte extract was subjected to sucrose density-gradient analysis on a 10%–50% gradient and fractionated as described (Gillian-Daniel et al. 1998). Thirty stage 40–41 embryos were lysed in basic lysis buffer (300 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl at pH 7.4) supplemented with 0.5% sodium deoxycholate and either 150 μg/mL cycloheximide, 250 μg/mL puromycin, or 25 mM EDTA. Lysates were fractionated on a 10%–50% sucrose gradient over a 65% cushion in basic lysis buffer and centrifuged for 2 h at 38,000 rpm in a TH461 ultracentrifuge rotor at 4°C. A_{254} profiles were analyzed on a density gradient-fractionation system (Teledyne Isco), and proteins were extracted from fractions by precipitation with 10% TCA prior to Western blot analysis.

Antibody generation

Antibodies were raised in New Zealand White rabbits with xDAZAP1-specific peptides

SGQQDFPFSQFGNAC and CGWTGQPPQTWQGYS,

conjugated to keyhole limpet hemocyanin. Bleeds were checked for reactivity, and the anti-serum from bleed 3 was affinity purified with antigenic peptides (CovalAb).

Protein extraction

Stage VI oocytes and embryos (stages 4–41) were lysed by mechanical disruption in RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2 mM DTT) supplemented with 2.5 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 25 mM NaF, 10 nM Calyculin A, 1 mM PMSF, 5 μg/mL Aprotinin, 5 μg/mL Leupeptin, 10 mM Pepstatin A, and 15 mM E64. Lysates were cleared by centrifugation at 13,000*g* for 5 min at 4°C, and the supernatants were retained. Protein concentrations were determined by Bradford assay, and equal amounts of protein were used for Western blot analysis.

Calf intestinal phosphatase treatment

Stage VI or progesterone matured oocytes were lysed by mechanical disruption in dephosphorylation buffer (25 mM HEPES at pH 7.6, 300 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton-X-100, 0.1 mM DTT, EDTA-free protease inhibitor tablets [Roche]). Equal amounts of protein lysate, as determined by Bradford assay, were incubated with calf intestinal phosphatase (CIP; 0.25 U/μg lysate) for 2 h at 37°C before boiling for 10 min at 90°C in protein loading buffer, followed by Western blot analysis.

Western blotting

Extracts or sucrose gradient fractions were resolved on either 10% SDS-PAGE gels or 4%–12% NuPAGE gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were probed with anti-xDAZAP1 (1:1000), anti-ePABP (1:3,000) (Wilkie et al. 2005), or anti-PABP1 (1:2000) (Wilkie et al. 2005), anti-tubulin (1:10,000; Sigma Aldrich), anti-phospho-ERK1/2 (1:2,000; Cell Signaling Technology), anti-ERK1/2 (1:1,000; Cell Signaling Technology), anti-polyhistidine (1:1,000; Sigma Aldrich), or RED2 anti-PABP1 (1:3000) antibody (gift from Simon Morley). Goat anti-rabbit IgG conjugated to horseradish peroxidase (1:150,000) secondary antibodies were used (Sigma Aldrich) and detected by enhanced chemiluminescence (Amersham/Thermo Fisher).

Expression and purification of recombinant proteins

Five hundred milliliter cultures of *E. coli* BL21(DE3), transformed with pET-hPABP1 or pMS2-hDAZAP, were induced at an optical density (600 nm) of 0.4, with 1 mM IPTG for 4 h at 22°C. Cells were lysed in 5 mL of lysis buffer (1× BugBuster [Novagen], 50 mM Tris-HCl at pH 8, 100 mM NaCl, 1 kU/mL Lysozyme, 25 U/mL Benzonase, EDTA-free protease inhibitor tablets [Roche]) for 20 min at room temperature. Clarified lysate was incubated with 500 μL Ni-NTA agarose (Qiagen) for 3 h at 4°C, washed six times with 10 mL wash buffer (50 mM Tris-HCl at pH 8, 300 mM NaCl, 20 mM imidazole, 0.2% NP40, EDTA-free protease inhibitor tablets [Roche]), and incubated in 500 μL elution buffer (50 mM Tris-HCl at pH 8, 100 mM NaCl, 0.05% NP40, 300 mM imidazole, 30% glycerol, EDTA-free protease inhibitor tablets [Roche]) for 10 min at 4°C with gentle agitation. The eluate was collected and stored at −80°C. Proteins were resolved on 4%–12% NuPAGE gels (Invitrogen) and either stained with GelCode Blue (Pierce) or quantified using SyproRuby (Invitrogen) alongside BSA standards, followed by scanning with a Typhoon Variable Mode Imager and quantification using ImageQuant software (GE Healthcare).

Protein–protein interactions

Yeast two-hybrid analysis was performed using the strain L40 as described (Gray et al. 2000). For Flag–pull-down assays, 30 pmol of purified recombinant hPABP1 or hDAZAP1 was incubated with 3 pmol of purified Flag-tagged human eIF4G and 30 μL anti-Flag M2 Affinity Gel (Sigma-Aldrich), in binding buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM DTT, 0.5% NP40, EDTA-free protease inhibitor tablets [Roche]) for 3 h at 4°C with agitation, washed six times in 1 mL binding buffer, and boiled for 10 min in 30 μL protein loading buffer. Samples were subjected to Western blot analysis using an anti-polyhistidine antibody (Sigma-Aldrich).

Bioinformatic analysis

Alternate forms of hDAZAP1 were identified in EnSEMBL and the alternative C-terminal exon of DAZAP1-202 (ENSP00000337132) was used to blastp search nonredundant protein sequences (nr), retrieving a sequence from *X. laevis* AAH77252.1. The human and *X. laevis* splice variants were aligned using color align conservation in the sequence manipulation suite (Stothard 2000).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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